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(54) CHOLESTEROL ASSAY

(71) We, NATIONAL RESEARCH DEVELOPMENT CORPORATION, a British corporation established by statute of Kingsgate House, 66/74 Victoria Street, London S.W.1., do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to the assay of cholesterol in liquids and particularly biological fluids such as serum.

The assay of total cholesterol, in its role as an indicator of atherolsclerosis and incipient coronary heart disease, now constitutes about 3% of the total number of tests performed in the average clinical chemistry laboratory. In Britain at the present time this represents about 1½ million cholesterol assays per year.

Cholesterol is currently assayed by the Liebermann-Burchard reaction which involves the use of highly corrosive and viscous reagents and presents many obstacles to automation.

It is an object of the present invention to provide an assay for cholesterol which does not have the disadvantages of the Liebermann-Burchard reaction and which can be readily automated.

Many *Nocardia* species are capable of metabolising cholesterol and in particular Stadtman et al (J.Biol. Chem. (1954) *206* 511—523) has disclosed that a soil Mycobacterium is capable of oxidising cholesterol to Δ^4-cholestenone. The so-called "cholesterol dehydrogenase" responsible for this reaction can be obtained in a cell-free form of low activity. The production of this "cholesterol dehydrogenase" from the same soil *Mycobacterium* as a slightly purer soluble enzyme preparation and the determination of the activity of the enzyme is described by Stadtman in Methods in Enzymology (1955) *1* 678—681 but the soluble enzyme is still of low activity and no purification was achieved.

The present invention provides an enzymic method for assaying for cholesterol in a liquid, in particular a biological fluid such as serum or plasma, which can readily be automated and which obviates the difficulties of the Libermann-Burchard reaction. The assay methods described are not, however, limited to biological fluids and can be applied quite generally for determining the amount of cholesterol present in any industrial or food product or in any industrial process where cholesterol assay may be considered necessary or desirable.

The invention provides a method of assaying for cholesterol in a liquid which comprises incubating the liquid with an enzyme preparation capable of oxidising the cholesterol into Δ^4-cholestenone and hydrogen peroxide and determining the amount of cholesterol present by measuring the amount in which hydrogen peroxide or Δ^4-cholestenone is formed or the amount in which oxygen is absorbed in the reaction.

The invention also provides a method of assaying for cholesterol in a liquid which comprises incubating the liquid with an enzyme preparation having cholesterol oxidase activity derived from a cholesterol oxidase producing micro-organism of the *Mycobacterium rhodocrous* group e.g. *Nocardia* species NCIB 10554 or NCIB 10555, and determining the amount in which hydrogen peroxide or Δ^4-cholestenone is formed in the cholesterol oxidase reaction or the amount in which oxygen is absorbed in the cholesterol oxidase reaction. Although any enzyme preparation having cholesterol oxidase activity, i.e. which is capable of oxidising cholesterol to Δ^4-cholestenone and hydrogen peroxide, can be used according to the invention, the preparation is pre-

ferably derived from a cholesterol oxidase producing micro-organism of the *Mycobacterium rhodocrous* group.

The enzyme preparation is most preferably derived from tw micro-organisms which are referred to as "rough" and "smooth" strains and have been given the numbers NCIB 10554 and NCIB 10555 respectively by the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen. The micro-organisms have also been deposited with the Agricultural Research Service of the United States Department of Agriculture as the ARS Culture Collection Investigations Fermentation Peoria, Illinois, U.S.A. where they have been given the numbers NRRL 5635 and NRRL 5636.

Full details of these organisms are given in our copending application No. 44095/72 (Serial No. 1,385,319). This application describes and claims enzyme preparations derived from a cholesterol oxidase producing micro-organism of the *Mycobacterium rhodocrous* group, preferably *Nocardia* species NCIB 10554 or NCIB 10555, and having a cholesterol oxidase specific activity of at least 1 unit per 5 mg of protein nitrogen.

The enzyme preparations according to Application 44095/71 (Serial No. 1,385,319) are preferred for use according to the present invention.

The enzyme preparation may be in liquid form or in solid, e.g. freeze-dried form. When the preparation is in solid form it should be reconstituted with buffer into a liquid form before it can be used in an assay. The enzyme preparation preferably has a cholesterol oxidase specific gravity of at least 1 unit per 5 mg of protein nitrogen.

The minimum potency of the liquid cholesterol oxidase preparation depends on the assay method for which it is to be used. Thus in the case of a preparation which is to be used for the assay of cholesterol by the fluorimetric determination of hydrogen peroxide produced the potency need only be relatively small, a potency of 10^{-2} units/ml of liquid preparation being sufficient.

The assay for cholesterol can also be carried out by using non-fluorimetric methods for the determination of hydrogen peroxide, e.g. colorimetric methods, or by measuring the amount in which Δ^4-cholestenone is formed or oxygen is consumed in the cholesterol oxidase reaction. In this case a potency of the cholesterol oxidase in the liquid preparation should preferably be at least 10^{-1} units/ml.

Particularly for use in automated analysis it is preferred that the enzyme preparation have a cholesterol oxidase specific gravity of at least 1 unit per 50 mg protein nitrogen and when made up in liquid form have a potency of at least 0.5 units/ml.

When the assay depends on the determination of hydrogen peroxide the presence of catalase in the enzyme preparation reduces the sensitivity of the assay. Generally a catalase activity of less than 10% of the cholesterol oxidase activity i.e. less than 10^{-1} units of catalase activity per unit of cholesterol oxidase activity, is tolerable and preferably for the preparation should have a catalase activity of less than 1% of the cholesterol oxidase activity, i.e. less than 10^{-2} units of catalase activity per unit of cholesterol oxidase activity.

The catalase activity of the enzyme preparation depends on the method by which it has been prepared and when the preferred method of Application No. 44095/71 (Serial No. 1,384,319) using a surface active agent is used the preparation contains only small amounts of catalase which do not reduce the sensitivity of the assay. However if other methods are used and the preparation does include catalase this can either be removed in the purification step or inhibited with a catalase inhibitor, such as an azide, e.g. sodium azide.

As used herein one unit of cholesterol oxidase activity is defined as that activity which will oxidise 1 μ mol (10^{-6} mol) of cholesterol to Δ^4-cholestenone and hydrogen peroxide per minute at 30°C and pH7. One unit of catalase activity is defined as that activity which will convert 1 μ mol of hydrogen peroxide to water and oxygen per minute at 25°C and pH7.

When the enzyme preparation is prepared according to the preferred method of Application No. 44095/71 (Serial No. 1,385,319) using a surface active agent, e.g. Triton X-100 (Triton is a Trade Mark), the surface active agent may remain in the cholesterol oxidase solution. In the assay method as described below it has been found that the presence of Triton X-100 may be advantageous but that the optimum concentration in the assay mixture is about 0.25%, higher concentrations being inhibitory. The enzyme preparation should thus not have a level of surface active agent greater than that which produces an acceptable level in the assay mixture.

As referred to above, in the case of a fluorimetric determination of hydrogen peroxide the enzyme preparation should have a potency of at least 10^{-2} units/ml to provide a result in a short enough time for an assay to be useful in practice and particularly where the analysis is automated the preparation should have a potency of at least 10^{-1} and preferably 0.5 units/ml.

In the case of a non-fluorimetric assay the preparation should have a potency of at least 10^{-1} units/ml and preferably for automated analysis at least 0.5 units/ml. The preparation may be prepared with a higher potency for example 5 units/ml or more but at higher levels of potency will generally be diluted with buffer before use in an assay.

In all cases the preparation preferably has a cholesterol oxidase specific activity of at least 1 unit per 5 mg. protein nitrogen. At higher levels of protein nitrogen the amount of protein present may make the assay solution too viscous or interfere in the assay. A specific activity of at least 1 unit per 50 μg protein nitrogen is most preferred. A particularly suitable enzyme preparation has been found to be an aqueous preparation with a specific activity of 1 unit of cholesterol oxidase activity per 28 μg protein nitrogen, a potency of 5 units/ml and containing 3% v/v Triton X-100. This preparation may be diluted for use in an assay, for example with 0.01M of phosphate buffer, so that for automated analysis it contains 0.5 units/ml or for manual analysis 0.1 units/ml.

The enzyme preparation having cholesterol oxidase activity need not be in aqueous form and it may be, for example, in the form of a freeze-dried powder. In addition to the preparation of a soluble lyophilised powder, the enzyme can be present as a concentrated, buffered solution or in suspension with ammonium sulphate (with or without added buffer).

Some catalyst activity will generally be present in the enzyme preparation together with cholesterol oxidase and the amount of catalase activity which can be tolerated in the enzyme preparation depends upon the assay method to be used, the preferred limits having already been given. The catalase activity of the preparation is important where the assay involves measuring the amount of H_2O_2 produced. As described in more detail below some assay methods in which H_2O_2 is determined include the use of peroxidase and in some cases it is possible to overpower limited amounts of catalase with peroxidase. In addition it is possible to inhibit any concentration of catalse likely to be found in the enzyme preparation with an inhibitor for example sodium azide.

The effect of catalase on the sensitivity of an assay can be demonstrated as follows:

A catalase solution was prepared by dissolving 20 μl of a crystalline suspension of catalase in 50 ml 0.05 M phosphate buffer pH 7.0.

0.5 ml of this catalase solution was found to break down 7.04 μM H_2O_2 (in a final volume of 2.5 ml) in 2.0 minutes. On this basis the catalase activity of the solution can be said to be 7.04 units per ml at pH 7.0 and 25°C.

Varying amounts of this catalase solution were introduced into a cholesterol assay system containing 0.5 units of cholesterol oxidase in 2.0 ml and the reduction in sensitivity of the assay observed.

Units catalase added	% reduction in sensitivity
0.704	95 %
0.352	66.4%
0.074	34.3%

However, the inclusion of 0.1% sodium azide in the reaction mixture completely inhibited even the highest level of catalase, thus giving 100% sensitivity even in the presence of catalase.

Catalase can, however, generally be removed from the enzyme preparation or at least reduced in amount, for example by chromatography on DEAE cellulose.

The method according to the invention can be used to assay free or total cholesterol in serum. Cholesterol is, of course, never free in plasma in the true sense as it is always combined with phospholipids, proteins and triglycerides in soluble lipoprotein complexes. Incorporation of a surface active agent in the assay mixture, however, causes dissolution of these complexes, under gentle conditions, allowing all the free cholesterol to be enzymically oxidised. The estimation of free cholesterol may be useful for screening purposes and may, in fact, obviate the need to measure total cholesterol.

Cholesterol also occurs in serum in the form of esters and, if total cholesterol

is to be assayed, the cholesterol bound in this way must first be brought into a form in which it can be attacked by cholesterol oxidase by hydrolysing the esters, for example by reaction with alcoholic potassium hydroxide. Reaction with 1.0 N KOH at 75°C effects rapid hydrolysis without coagulation of protein.

To conduct the assay the optionally hydrolysed fluid is then incubated with cholesterol oxidase and the reaction

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{cholest-4-en-3-one} + H_2O_2$$

is preferably allowed to go to completion. The amount of cholesterol present is determined by measuring the oxygen uptake or the amount in which at least one of the products is formed. Under suitably standardised conditions it is possible to estimate the amount of cholesterol present from the amount of a product formed or the amount of oxygen used in a given time even if the reaction has not been allowed to go to completion.

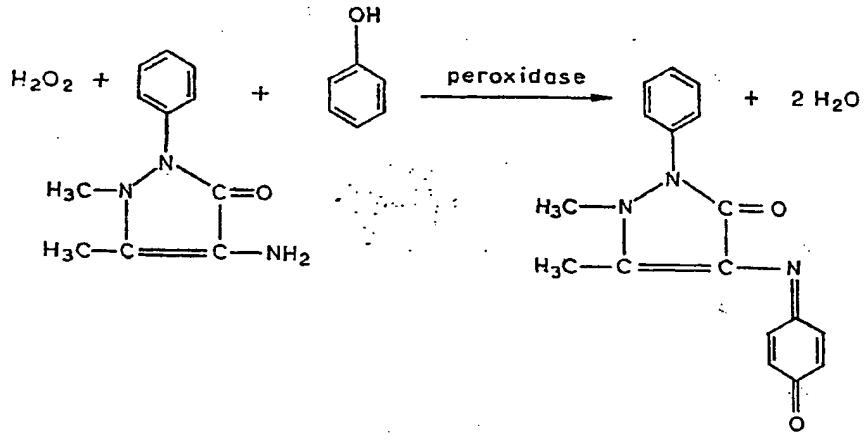
As mentioned above, when hydrogen peroxide is estimated fluorimetrically the liquid enzyme preparation should preferably have a cholesterol oxidase specific activity of at least 1 unit 5 mg of protein nitrogen and a potency of at least 10^{-2} units/ml. With any other estimation technique, i.e. measuring the amount of hydrogen peroxide colorimetrically, measuring the oxygen uptake, e.g. with an oxygen electrode, or measuring the amount of Δ^4-cholestenone produced either directly by its carbonyl absorption or by forming a derivative which is measured colorimetrically, the liquid enzyme preparation should preferably have a cholesterol oxidase specific activity of at least 1 unit per 5 mg of protein nitrogen and a potency of at least 10^{-1} units/ml.

The assay may, of course, either be carried out as an individual test on a single sample or a plurality of samples may be tested on an automated basis. The preferred enzyme preparation for automated analysis is one with a cholesterol oxidase specific activity of at least 1 unit per 50 µg of protein nitrogen and a potency of at least 0.5 unit/ml.

Preferably the amount of hydrogen peroxide produced is measured by a system which comprises a chromogenic reagent or reagents capable of undergoing a colour change in the presence of hydrogen peroxide, the amount of hydrogen peroxide present being measured by colorimetrically measuring the colour change of the chromogenic reagent or reagents.

In one preferred method the hydrogen peroxide produced is measured by means of quadrivalent-titanium and xylenol orange which react to form a stable red colour with hydrogen peroxide (Tammes & Nordschow, Amer, J. Clin. Path. (1968), *49*, 613). The amount of hydrogen peroxide produced is measured by the intensity of the colour.

In another preferred method the amount of cholesterol present is determined by measuring the amount of hydrogen peroxide produced by means of the reaction with 4-amino-phenazone in the presence of excess phenol and peroxidase, the reaction being as follows:



Thus the preferably hydrolysed extract is reacted with cholesterol oxidase in the

presence of peroxidase, 4-amino-phenazone and phenol and the optical density is measured at 510 nm. It can be shown that there is a linear relationship between the ΔOD (difference in optical density) between a test solution and a control containing no cholesterol and the amount of cholestenone produced. The cholesterol oxidase, peroxidase, 4-aminophenazone and phenol can be made up into a single reagent and used in this form.

In a typical assay according to this method 0.1 ml of serum may be added to 1.0 ml alcoholic KOH (1 N) and incubated at 75°C for 5 minutes. 0.1 ml of the saponified extract may then be added to 2.5 ml of a cholesterol oxidase/peroxidase/4-aminophenzone/phenol reagent and the mixture incubated at 30 to 37°C for 5 minutes and the colour read at 510 nm.

In this test a 0.1% solution of cholesterol may produce a turbidity when added to the enzyme reagent giving an optical density of about 0.05. A 0.1% solution of cholestenone treated in the same manner may also produce a turbidity giving an optical density of about 0.08. These conditions would only be encountered in an extremely abnormal serum containing 1,000 mg % cholesterol but the presence of a surface active agent such as 0.1% Triton X-100 in the enzyme reagent prevents this turbidity, i.e. neither the substrate nor the product produce during the reaction a colloidal suspension which would interfere with spectrophotometry.

Any other suitable reaction of hydrogen peroxide may be used to measure the amount produced and hence the amount of chloesterol in the serum. Examples of such reactions with the references where a fuller description of the reaction may be found are as follows:

(1) 2,6-dichlorophenol indophenol can be used as the oxygen acceptor instead of 4-aminophenazone in a coupled peroxidase reaction (Clark & Timms, Proc. Assoc. Clin. Biochem. (1968) 5, 61).

(2) Hydrogen peroxide can be reacted with guaiacum in the presence of peroxidase to give a blue product (Morley, Dawson & Marks, Proc. Assoc. Clin. Biochem. (1968) 5, 42).

(3) Iodine is liberated from potassium iodide on reaction with hydrogen peroxide and the liberated iodidine can then be reacted to form a pink colour with diethyl-*p*-phenylene-diamine (Thompson, Clin. Chim. Acta. (1969) 25, 475).

(4) Iodine is liberated from iodide by reaction with hydrogen peroxide and polyvinyl pyrrolidine is used to shift the absorption of iodine from the near n.v. towards the visible where its absorption can be read at 470 nm (Wate and Marbach, Clin. Chem. (1968) *14*, 548).

(5) Under the action of peroxidase hydrogen peroxide oxidises homovanillic acid to a highly fluorescent product in alkaline solution (Protein does not interfere with this reaction at a 40-fold dilution of plasma). The product can be measured fluorimetrically (Phillips & Elevitch, Amer. J. Clin. Path. (1968) *49*, 622).

(6) Hydrogen peroxide reacts with iodine in the presence of a molybdenum (IV) catalyst. If a known excess of thiosulphate is used to reduce the iodine as it is preduced residual thiosulphate can be titrated colorimetrically with iodine. (Simon, Christian & Purdy, Clin. Chem. (1968) *14* 463).

Although measuring the hydrogen peroxide produced is the preferred method of determining the amount of cholesterol present in the biological fluid being assayed it is also possible to use other techniques to obtain the required assay. For example, the oxygen uptake in the cholesterol oxidase reaction can be measured using an oxygen electrode by the technique described by Updike & Hicks in Nature, Lond. (1967) *214*, 986 and Makin & Warren in Clin. Acta. (1970) *29*, 493. Alternatively the oxidation product Δ^4-cholestenone can be measured for example as its 2,4-diphenyl hydrazone or iso-nicotinic acid hydrazine, or if a sufficiently pure enzyme is used in a concentration that exhibits low absorption at 240 nm (or if the enzyme is immobilised) the cholesterol can be assayed by the increase in absorption at 240 nm due to the Δ^4-cholestenone formed by enzymic oxidation.

As mentioned above cholesterol assay can either be conducted on an automated basis where a large number of assays are conducted in succession or small numbers of samples can be analysed individually. The reagents required for the assay will depend upon the particular method but when a H_2O_2 determination is used as the basis of the assay the reagents will generally comprise (1) the cholesterol oxidase preparation, and (2) one or more other reagents required for determining the amount of H_2O_2 produced. Some of the other reagents may be compatible with the cholesterol oxidase and may be incorporated into the enzyme preparation to give a combined reagent. In the case of assay methods requiring the presence of peroxidase this can be included with the cholesterol oxidase to form a combined liquid or freeze

dried enzyme preparation. In the case of a freeze dried preparation this is reconstituted before use by addition of buffer. In all cases the reagents are generally supplied in a concentrated form and diluted immediately before use. A reagent for hydrolysing the biological fluid is also required when total cholesterol is to be measured. Where this is alcoholic potassium hydroxide this will generally be supplied by the user as will buffer and other reagents required for dilution.

For automated analysis the cholesterol oxidase preparation is supplied either in concentrated or freeze dried form to be diluted or reconstituted before use. If required by the analysis the preparation may also contain peroxidase. The other reagents will generally be supplied by the user.

Where individual tests are to be performed kits of the reagents can be supplied.

The invention thus also provides a kit for use in assaying the amount of chloesterol in a liquid comprising in association,

i) an enzyme preparation capable of oxidising cholesterol to Δ^4-cholestenone and hydrogen peroxide; and

ii) at least one reagent which is capable of being used in the determination of the amount in which hydrogen peroxide or Δ^4-cholestenone is formed.

Preferably component (i) is an enzyme preparation derived from a cholesterol oxidase producing micro-organism of the *Mycobacterium rhodocrous* group, e.g. *Nocardia* species NCIB 10554 or NCIB 10555. Preferably the enzyme preparation has a cholesterol oxidase specific activity of at least 1 unit per 5 mg of protein nitrogen.

Component (i) is thus the enzyme preparation generally in a form more concentrated than required in the test to be diluted with buffer by the user. The enzyme preparation may be in freeze-dried or in concentrated liquid form. The enzyme preparation may include peroxidase when this is required for the test or any other reagent required in the final assay which is compatible with the cholesterol oxidase.

The kit will, of course, contain each of the components in the amount required for the same defined number of tests. For example the kit may contain sufficient enzyme and other reagents for say 12 tests. Each reagent may be supplied in a single amount, e.g. in a bottle, the amount required for an individual test being extracted from this single amount.

Alternatively the kit may contain unit doses of the enzyme component, each containing at least 0.05 units, preferably at least 0.5 units, of cholesterol oxidase activity, i.e. enough for a single test. For example the unit doses of enzyme preparation may be provided in freeze-dried or concentrated form in individual vials to be reconstituted with buffer before each test.

Component (ii) of the kit is, where hydrogen peroxide is to be determined colorimetrically, usually an oxygen acceptor such as 4-aminophenazone and phenol or xylenol orange and quadrivalent titanium. These other reagents can be supplied in bottles either ready for use or in concentrated form to be diluted by the user.

The kit may also include as a further component (iii) at least one reagent capable of being used in hydrolysing the serum. This component will, of course, only be needed where total cholesterol is to be measured.

The user will generally supply the buffer required for dilution and any other common reagents which may be required such as solvents for extraction or dilution. Where the assay is for total cholesterol and hydrolysis is carried out with an alkali such as alcoholic KOH an acid reagent, e.g. dilute HCl or H_2SO_4, is required for neutralisation of the reagent used for hydrolysis and this acid reagent may be included in the kit. In cases where Δ^4-cholestenone is determined directly by its carbonyl absorption and there is no colour reagent this acid reagent may be the only other component of the kit apart from the enzyme preparation. If required the kit may include one or more standard cholesterol solutions for standardising the assay.

The invention is illustrated by the following examples although it is to be understood that they do not limit the invention in any way. In the examples the cholesterol oxidase is that produced according to the example of our copending Application No. 44095/71 (Serial No. 1,385,319).

Example 1

Assay of Free Cholesterol in Serum

Principle

Cholesterol oxidase oxidises cholesterol to Δ^4-cholestenone with the simultaneous production of hydrogen peroxide. The hydrogen peroxide produced is chelated with xylenol orange and quadrivalent titanium. The absorption of this red coloured complex is measured at 550 nm.

Reagents

1. 0.01 M phosphate buffer pH 7.0 containing 0.10 g % sodium azide.
2. Cholesterol oxidase solution 5 units/ml (1 ml oxidises 5 μM cholesterol per minute at pH 7.0 and 30°C) contains approx. 3.0 % v/v Triton X-100.
3. Working enzyme solution—5 ml cholesterol oxidase solution is added to 45 ml of the 0.01 M phosphate buffer.
4. Sulphuric acid 2N—56 ml concentrated sulphuric acid is added to distilled water and diluted to one litre.
5. Stock titanium 0.001 M—0.08 g titanium dioxide is placed in a 25 ml conical flask and 0.5 g ammonium sulphate and 2.5 ml concentrated sulphuric acid are added. This mixture is heated on a hot plate until all of the material is dissolved and is colourless. The solution is cooled and made up to one litre with distilled water.
6. Stock xylenol orange 0.001 M—0.76 g xylenol orange is dissolved in distilled water and diluted to one litre.
7. Combined colour reagent—Add one volume of stock titanium to 0.5 volume of 2N sulphuric acid, mix. Add one volume of stock xylenol orange and 0.5 g (polyoxyethylene lauryl ether) per 1 litre of reagent.
8. Cholesterol standards containing up to 5.0 mM per litre. Prepared by dissolving pure dry cholesterol (BDH Biochemical Standard) in isopropyl alcohol.

Method

100 μl serum or standard are added to 2.0 ml working enzyme solution and incubated at 37°C for 5 minutes. 1.0 ml of the combined colour reagent is then added and the mixture incubated for a further 5 minutes at 37°C.

Blanks are prepared by adding serum or standard directly to a mixture of 2.0 ml working enzyme solution and 1.0 ml combined colour reagent.

The optical density of the test solution is read against its blanks at 550 nm.

Example 2
Assay of Total Cholesterol in Serum

Principle

Cholesterol is released from lipoprotein complexes and hydrolysed from its esters by alkaline hydrolysis. Following neutralisation of the hydrolysate the cholesterol is enzymically oxidised to Δ^4-cholestenone. Δ^4-cholestenone is then extracted from the reaction mixture and its absorption at 236 nm is measured.

Reagents

1. 0.05 M phosphate buffer at 7.0.
2. Cholesterol oxidase—Stock solution containing 5.0 units/ml and 3.0 % v/v Triton X-100.
3. Working enzyme—5.0 ml of the cholesterol oxidase stock solution is added to 45 ml of the 0.05 M phosphate buffer pH 7.0 (1).
4. Ethanolic KOH (IN)—A "Volucon" ampoule (May and Baker Ltd—Volucon is a Trade Mark) to make 1 litre 1 N KOH is diluted to 100 ml with distilled water and made up to 1 litre with absolute alcohol.
5. 0.083 N Hydrochloric Acid containing 0.3 % v/v Triton X-100.
6. Cholesterol Standards up to 12.9 mM (500 mg %). Prepared by dissolving pure dry cholesterol (BDH Biochemical Standard) in isopropyl alcohol.
7. Cyclohexane.

Method

0.2 ml serum or standard is added to 1.0 ml ethanolic KOH and the mixture incubated for 5 minutes at 75°C in stoppered tubes.

0.1 ml of the saponified extract is now added to 1.0 ml 0.083 N HCl containing 0.3 % Triton X-100. The solution is mixed before adding 1.0 ml of the working enzyme solution. After addition of the cholesterol oxidase the reaction mixture is incubated at 37°C for 10 minutes.

5.0 ml ethanolic KOH are now added and the Δ^4-cholestenone formed during the reaction is extracted into 3.0 ml cyclohexane.

Blanks are run in a similar fashion except that the working enzyme solution is not added until after the addition of the ethanolic KOH.

The optical density of an extract is read against its blank at 236 nm.

Example 3

Kit Formulation for Assay or Free Cholesterol in Serum

Principle

Cholesterol oxidase oxidises cholesterol to Δ^4-cholestenone with the simultaneous production of hydrogen peroxide. The hydrogen peroxide produced is chelated with xylenol orange and quadrivalent titanium. The absorption of this red coloured complex is measured at 550 nm.

Reagents

1. 0.01 M phosphate buffer pH 7.0 containing 0.10 g % sodium azide.
2. Cholesterol oxidase solution 5 units (1 ml oxidises 5 μM cholesterol per minute at pH 7.0 and 30°C) containing 3.0 % v/v Triton X-100.
3. Working enzyme solution—5 ml cholesterol oxidase solution is added to 41 ml of the 0.01 M phosphate buffer (1).
4. Sulphuric acid 2N—56 ml concentrated sulphuric acid is added to distilled water and diluted to our litre.
5. Stock titanium 0.001 M—0.08 g titanium dioxide is placed in a 25 ml conical flask and 0.5 ammonium sulphate and 2.5 ml concentrated sulphuric acid are added. This mixture is heated on a hot plate until all the material is dissolved and is colourless. The solution is cooled and made up to one litre with distilled water.
6. Stock xylenol orange 0.001 M—0.76 g xylenol orange is dissolved in distilled water and diluted to one litre.
7. Combined colour reagent—One volume of stock titanium is added to 0.5 volume of 2N sulphuric acid and mixed. One volume of stock xylenol orange and 0.5 g Brij-35 (polyoxyethylene lauryl ether) per litre of reagent is then added.
8. A cholesterol standard containing up to 3.0 nM per litre is prepared by dissolving pure dry cholesterol (BDH Biochemical Standard) in isopropyl alcohol.

The kit for 20 tests includes two reagents A and B and a standard cholesterol solution (reagent 8 above).

Reagent A—80 mls Cholesterol oxidase solution prepared as reagent (3) above.

Reagent B—40 mls combined colour reagent prepared as reagent (7) above.

Method

Add 100 μl serum or cholesterol standard to 2.0 ml reagent A and incubate at 37°C for 10 minutes.

Add 1.0 ml reagent B to the reaction mixture and continue incubation at 37°C for a further five minutes.

Blanks are prepared by adding serum or cholesterol standard directly to a mixture of 2 mls reagent A and 1.0 ml reagent B and incubating for five minutes at 37°C.

The optical densities of the test solutions are read against their corresponding blanks at 550 nm.

Calculation of Results

$$\text{mM cholesterol for litre test} = \frac{3.0 \times OD_{550} \text{ test}}{OD_{550} \text{ standard.}}$$

Example 4

Kit Formulation for Assay of Total Cholesterol in Serum

Principle

Cholesterol is released from lipoprotein complexes and hydrolysed from its esters by alkaline hydrolysis. Following neutralisation of the hydrolysate the cholesterol is enzymically oxidised to Δ^4-cholestenone and its absorption at 236 nm is measured.

Reagents

1. 0.05 M phosphate buffer pH 7.0
2. Cholesterol oxidase—Stock solution containing 5.0 units/ml and 3.0 % v/v Triton X-100.
3. Working enzyme—5.0 ml of the cholesterol oxidase stock solution is added to 45 ml of the 0.05 M phosphate buffer pH 7.0 (1).
4. Ethanolic KOH (1N)—"Volucon" ampoule (May & Baker Ltd) to make 1 litre IN KOH is diluted to 100 ml with distilled water and made up to 1 litre with Absolute Alcohol.

5. 0.083 N Hydrochloric acid containing 0.3 % v/v Triton X-100.
6. Cholesterol Standard up to 12.9 mM (500 mg %) prepared by dissolving pure dry cholesterol (BDH Biochemical Standard) in isopropyl alcohol.
7. Cyclohexane.

A kit for 20 tests includes two reagents A and B and standard Cholesterol solution (reagent 6 above).
Reagent A—Cholesterol oxidase—80 ml working enzyme (reagent 3).
Reagent B—80 mls. 0.083 N hydrochloric Acid containing 0.3 % v/v Triton X-100 (reagent 5.)
Ethanolic KOH and cyclohexane are provided by the user, as the former is unstable and both are required in relatively large volumes.

Method

0.2 ml serum or standard is added to 1.0 ml ethanolic KOH and the mixture incubated for 5 minutes at 75°C in stoppered tubes.

0.1 ml of the saponified extract is now added to 1.0 ml 0.083 N HCl containing 0.3% Triton X-100 (Reagent A). The solution is mixed before adding 1.0 ml of the working enzyme solution (Reagent B). After addition of the cholesterol oxidase the reaction mixture is incubated at 37° for 10 minutes.

5.0 ml ethanolic KOH are now added and the Δ^4-cholestenone formed during the reaction is extracted into 4.0 ml cyclohexane.

Blanks are run in a similar fashion except that the working enzyme solution is not added until after the addition of the ethanolic KOH.

The optical density of the extracts is read against their blanks at 236 nm.

WHAT WE CLAIM IS:—

1. A method of assaying for cholesterol in a liquid which comprises incubating the liquid with an enzyme preparation capable of oxidising the cholesterol into Δ^4-cholestenone and hydrogen peroxide and determining the amount of cholesterol present by measuring the amount in which hydrogen peroxide or Δ^4-cholestenone is formed or the amount in which oxygen is absorbed in the reaction.

2. A method of assaying for cholesterol in a liquid which comprises incubating the liquid with an enzyme preparation having cholesterol oxidase activity which is derived from a cholesterol oxidase producing micro-organism of the *Mycobacterium rhodocrous* group and determining the amount in which hydrogen peroxide or Δ^4-cholestenone is formed in the cholesterol oxidase reaction or the amount in which oxygen is absorbed in the cholesterol oxidase reaction.

3. A method as claimed in Claim 2 in which the enzyme preparation is derived from *Nocardia* species NCIB 10554 or NCIB 10555.

4. A method as claimed in any of Claims 1 to 3 in which the enzyme preparation having a cholesterol oxidase specific activity of at least 1 unit per 5 mg of protein nitrogen and a potency of at least 10^{-2} units/ml and the amount of hydrogen peroxide produced is measured by a fluorimetric method.

5. A method as claimed in any of Claims 1 to 3 in which the enzyme preparation is a liquid enzyme preparation having a cholesterol oxidase specific activity of at least 1 unit per 5 mg of protein nitrogen and a potency of at least 10^{-2} units/ml.

6. A method as claimed in Claim 5 in which the amount of hydrogen peroxide produced is measured colorimetrically the oxygen uptake is measured using an oxygen electrode or the amount of Δ^4-cholestenone produced is measured either directly by its carbonyl absorption or a derivative of Δ^4-cholestenone is formed which is measured colorimetrically.

7. A method as claimed in Claim 6 in which the amount of hydrogen peroxide produced is measured by a system which comprises a chromogenic reagent or reagents capable of undergoing a colour change in the presence of hydrogen peroxide, the amount of hydrogen peroxide being measured by colorimetrically measuring the colour change in the chromogenic reagent or reagents.

8. A method as claimed in any of Claims 1 to 7 in which the enzyme preparation is a liquid enzyme preparation having a cholesterol oxidase specific activity of at least 1 unit per 50 μg of protein nitrogen and a potency of at least 0.5 unit/ml.

9. A method as claimed in any of Claims 1 to 8 in which the liquid is serum.

10. A method as claimed in Claim 9 in which for the assay of total cholesterol, the serum is first hydrolysed.

11. A method as claimed in any of Claims 1 to 10 carried out as an individual test on a single sample.

12. A method as claimed in any of Claims 1 to 10 in which a plurality of samples are tested on an automated basis.

13. A method as claimed in Claim 1 substantially as hereinbefore described.

14. A method of assaying for cholesterol in a liquid substantially as hereinbefore described with reference to any of Examples 1 to 4.

15. A kit for use in assaying the amount of cholesterol in a liquid comprising in association,

i) an enzyme preparation capable of oxidising cholesterol to Δ^4-cholestenone and hydrogen peroxide; and

ii) at least one reagent which is capable of being used in the determination of the amount in which hydrogen peroxide or Δ^4-cholestenone is formed.

16. A kit as claimed in Claim 15 in which the enzyme preparation (i) has a cholesterol oxidase specific activity of at least 1 unit per 5 mg of protein nitrogen.

17. A kit as claimed in Claim 15 in which the enzyme preparation (i) is derived from a cholesterol oxidase producing micro-organism of the *Mycobacterium rhodocrous* group.

18. A kit as claimed in Claim 17 in which the enzyme preparation (i) is derived from *Nocardia* species NCIB 10554 or NCIB 10555.

19. A kit for use in assaying the amount of cholesterol in a liquid comprising in association

i) an enzyme preparation which is derived from a cholesterol oxidase producing micro-organism of the *Mycobacterium rhodocrous* group and having a cholesterol oxidase specific activity of at least 1 unit per 5 mg of protein nitrogen; and

ii) at least one reagent which is capable of being used in the determination of the amount in which hydrogen peroxide or Δ^4-cholestenone is formed in the cholesterol oxidase reaction.

20. A kit as claimed in Claim 19 in which the enzyme preparation (1) is derived from *Nocardia* species NCIB 10554 or NCIB 10555.

21. A kit as claimed in any of Claims 15 to 20 in which the enzyme preparation (i) comprises an aqueous solution of the enzyme having a potency of at least 10^{-2} units/ml.

22. A kit as claimed in any of Claims 15 to 21 in which component (ii) comprises at least one reagent capable of taking part in a reaction by means of which hydrogen peroxide can be determined fluorometrically.

23. A kit as claimed in any of Claims 15 to 21 in which component (ii) comprises at least one reagent capable of taking part in a reaction by means of which hydrogen peroxide can be determined colorimetrically.

24. A kit as claimed in any of Claims 15 to 23 in which the enzyme preparation (i) has a cholesterol oxidase specific activity of at least 1 unit per 50 mg of protein nitrogen and, if in the form of an aqueous solution of the enzyme, has a potency of at least 0.5 unit/ml.

25. A kit as claimed in any of Claims 15 to 24 in which component (i) is a freeze-dried enzyme preparation to be made up to an aqueous preparation of the required potency with buffer.

26. A kit as claimed in any of Claims 15 to 25 which contains each of the components in the amount required for the same defined number of tests.

27. A kit as claimed in Claim 26 which contains a single amount of each component, the amount required for an individual test to be extracted from this single amount.

28. A kit as claimed in Claim 26 which contains unit doses of the enzyme component (i) each containing at least 0.05 units of cholesterol oxidase activity.

29. A kit as claimed in Claim 28 in which unit doses of the enzyme component (i) are provided in freeze-dried or concentrated form in individual vials to be reconstituted with buffer before each test.

30. A kit as claimed in any of Claims 15 to 29 for the assay of total cholesterol in serum which contains in addition to components (i) and (ii),

(iii) at least one reagent which is capable of being used in hydrolysing the serum.

31. A kit as claimed in any of Claims 15 to 30 which includes in addition at least one standard cholesterol solution.

32. A kit as claimed in Claim 15 substantially as hereinbefore described.

33. A kit for use in assaying for cholesterol in a liquid substantially as hereinbefore described with reference to either of Examples 3 and 4.

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